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## Partial characterisation of peptides inhibiting *Listeria* growth in two Alpine cheeses

Phuong Nguyen Thi · Coralie Dupas · Isabelle Adt ·  
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**Abstract** *Listeria monocytogenes*, agent of food-borne listeriosis, is a major concern in dairy industry. The aim of this study was to assess the occurrence of peptides inhibiting *Listeria* spp. growth in two traditional Alpine pressed-curd cheeses: Emmental de Savoie and Asiago d'Allevo, and to get further insights regarding the characteristics of these peptides. Water-soluble extracts of these two cheeses were ultrafiltered onto 10,000-g.mol<sup>-1</sup> cut-off filters to remove proteins and the corresponding filtrate was subsequently dialysed with 100–500-g.mol<sup>-1</sup> cut-off membranes to remove salt and organic acids. A decrease from 53±2% to 68±1% of *Listeria innocua* LRGIA01 cells growth after 12-h incubation at 30 °C was observed in the presence of lyophilised extracts of the four cheese samples which were analysed. A less pronounced inhibition of *L. monocytogenes* 162 strain cells growth by cheese extracts was observed under the same conditions. After ultrafiltration onto 1,000 g.mol<sup>-1</sup> cut-off membranes, all the extracts had almost the same anti-*L. innocua* activity, suggesting that most of antibacterial

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peptides had a molecular mass between 100–500 and 1,000 g.mol<sup>-1</sup>. The assay of the anti-*L. innocua* activity of fractions of Asiago cheese extracts separated by cation-exchange chromatography indicated that non-cationic peptides had a lower antibacterial activity. Taken together, these data suggest that most of antibacterial peptides present in Asiago cheese are low molecular mass and cationic peptides. A significant contribution of intact bacteriocins is thus unlikely; a contribution of either antimicrobial fragments of bacteriocins or caseins released by proteolytic enzymes during cheese ripening is more likely.

**Keywords** Cheese ripening · Antimicrobial peptides · Antilisterial activity · *Listeria innocua* · *Listeria monocytogenes*

## 1 Introduction

Fermentation is a traditional method for food preservation. This is one of the reasons why the shelf life of cheeses is longer than that of raw milk. In combination with water activity reduction resulting from draining of cheese whey, salt addition and drying during cheese ripening, the action of microorganisms present in cheese curd is well-known to contribute to cheese preservation. Most of the corresponding mechanisms (i.e. competition for nutrients, production of antimicrobial metabolites such as lactic acid) are well described. However, some additional less well established mechanisms, such as antimicrobial peptides release during cheese ripening, have been proposed to also play a role in cheese as well as in human health preservation. These antimicrobial peptides can be bacteriocins directly produced by lactic acid bacteria (Rodriguez et al. 1998) as well as antimicrobial fragments from milk proteins. The presence of casein fragments inhibiting the growth of food-borne pathogenic or food spoilage bacteria has increasingly been reported in recent years in several cheeses: Pecorino Romano, Canestrato Pugliese, Crescenza, Caprino del Piemonte (Rizzello et al. 2005), commercial Australian Cheddar (Pritchard et al. 2010) and Asiago (Lignitto et al. 2012). The liberation as well as the degradation into inactive fragments of these antimicrobial casein fragments during cheese ripening can result from the action of proteolytic enzymes from milk, rennet or microorganisms (Gobbetti et al. 2002; Silva and Malcata 2005). Rizzello et al. (2005) suggested that the absence of peptides inhibiting *L. innocua* DSM20649 growth in Parmigiano-Reggiano, Fossa and Gorgonzola cheeses is due to the higher level of proteolysis in these cheeses which led to their degradation. Since the presence of antimicrobial peptides may vary from one cheese sample to another, namely as a function of their proteolysis index, the anti-*Listeria* activity of peptides extracted from three Emmental de Savoie cheese samples was first assessed. The anti-*Listeria* activity of peptides extracted from one Asiago cheese sample (chosen for its high anti-*Listeria* activity) ripened for 5 months was also determined. The main goal of the present study was to get further insight in the structural characteristics of peptides inhibiting *Listeria* spp. growth present in hard cheeses: the contribution of peptides with a molecular mass lower than 1,000 g.mol<sup>-1</sup> to the cheese extracts anti-*Listeria* activity was thus determined and the anti-*Listeria* activity of different cation-exchange chromatography fractions of an Asiago cheese extract was assayed in order to assess the contribution of cationic peptides.

## 2 Material and methods

### 2.1 Cheeses

Three Emmental de Savoie (Protected Geographical Indication) (ripened for 97, 114 and 149 days) and one Asiago d'Allevo (Protected Designation of Origin) (ripened for 150 days) cheese samples were collected from different cheesemaking plants in France and Italy, respectively. These cheeses were made following traditional methods from raw milk using calf rennet and lactic acid bacteria starter cultures. Only one Asiago cheese sample was considered, since Lignitto et al. (2012) already studied the influence of the length of Asiago cheeses ripening time on their anti-*Listeria* activity. In the case of Asiago d'Allevo, a selected strain (T1/A-D, Bioagro Srl, Thiene, Vicenza, Italy) of *Streptococcus thermophilus* was used as starter (see Segato et al. 2007 for more details). Slices of approximately 250 g were cut from the centre to the surface of the cheeses (all the cheeses were cylindrical) and were stored at  $-18\text{ }^{\circ}\text{C}$  until their analysis. Prior to their analysis, the slices of each cheese were grated after the rinds were removed and discarded. Total nitrogen (TN) and water-soluble nitrogen (WSN) of cheeses was determined by the Kjeldahl method (AOAC 1984). Prior to WSN determination, the water-soluble fraction of cheeses was prepared as described in Bouton et al. (1993). The proteolysis index of cheeses was calculated as the WSN/TN ratio.

### 2.2 Preparation of cheese water-soluble extracts

Water-soluble extracts (WSEs) of cheeses were prepared according to a previously described procedure (Dupas et al. 2009), with the following modifications: the pH of the initial cheese mixture was adjusted to 2, to prevent further enzymatic degradation of peptides, and the final filtrates (obtained through  $10,000\text{ g}\cdot\text{mol}^{-1}$  cut-off polyethersulfone ultrafiltration membranes) were further dialysed for 48 h at  $4\text{ }^{\circ}\text{C}$  through a  $100\text{--}500\text{ g}\cdot\text{mol}^{-1}$  cut-off cellulose ester membrane (Spectra/Por<sup>®</sup>, Breda, NL) to remove lower molecular mass molecules (such as salt). Salt removal was checked using a chloride analyser (Sherwood Scientific, Cambridge, U.K.), pH was adjusted to 7.5 (pH for *Listeria* spp. optimal growth) using NaOH 3% (*w/v*) and the samples were freeze-dried and stored at  $-20\text{ }^{\circ}\text{C}$  until used.

The WSEs extracted from each of the four cheese samples were analysed by gel filtration chromatography (GFC). A Perkin Elmer (Norwalk, CT, USA) HPLC system consisting of a binary LC250 pump, a 235C diode array detector, a computer with TotalChrom Navigator<sup>®</sup> software and a Superdex<sup>®</sup> Peptide PE 7.5/300 column (Pharmacia Biotech, Uppsala, Sweden) were used. Samples (0.3 mg of each cheese WSE lyophilisate suspended in 0.1 mL of water) were loaded onto the column and eluted for 120 min with 30% (*v/v*) of 0.1% (*v/v*) trifluoroacetic acid (TFA) in acetonitrile and 70% (*v/v*) of 0.1% (*v/v*) TFA in water. The flow rate was  $0.25\text{ mL}\cdot\text{min}^{-1}$ , and detection was carried out at 215 nm. The Superdex<sup>®</sup> Peptide PE 7.5/300 column was calibrated with proteins, peptides, and free amino acids of different molecular masses (cytochrom c ( $12,500\text{ g}\cdot\text{mol}^{-1}$ ), aprotinin ( $6,511\text{ g}\cdot\text{mol}^{-1}$ ), substance P ( $1,347\text{ g}\cdot\text{mol}^{-1}$ ), angiotensin-converting enzyme inhibitor ( $1,101\text{ g}\cdot\text{mol}^{-1}$ ), Tyr-Lys ( $327\text{ g}\cdot\text{mol}^{-1}$ ), Tyr-Pro ( $296\text{ g}\cdot\text{mol}^{-1}$ ), Gly-Gly-Gly ( $189\text{ g}\cdot\text{mol}^{-1}$ ), L-Tyr ( $204\text{ g}\cdot\text{mol}^{-1}$ ) and L-Trp ( $181\text{ g}\cdot\text{mol}^{-1}$ )) as described in Dupas et al.

(2009). The proportion of molecules with a molecular mass between 200 and 1,000  $\text{g}\cdot\text{mol}^{-1}$  was calculated as the ratio between the area under curve (AUC) (plotting of 215-nm absorbance vs. time) of molecules eluted between 32.8 and 41.0 min (i.e. having a molecular mass lower than 1,000  $\text{g}\cdot\text{mol}^{-1}$ , as deduced from the GFC molecular mass vs. elution time calibration curve) and the total AUC for each cheese WSE and for each cation-exchange chromatography fraction of Asiago cheese extract (see below).

Moreover, a part of cheese WSEs lyophilisates were resuspended in water at a concentration of 40  $\text{mg}\cdot\text{mL}^{-1}$  of powder and further fractionated through a 1,000  $\text{g}\cdot\text{mol}^{-1}$  cut-off membrane (Microsep, Pall Gelman laboratories, Saint-Germain-en-Laye, France). The permeates (containing compounds with a molecular mass lower than 1,000  $\text{g}\cdot\text{mol}^{-1}$ ) were mixed with an equal volume of double-strength (2 $\times$ ) Brain Heart Infusion (BHI) broth and used to assess their respective effects on the growth of *Listeria* spp.

### 2.3 Fractionation of cheese extracts by cation-exchange liquid chromatography

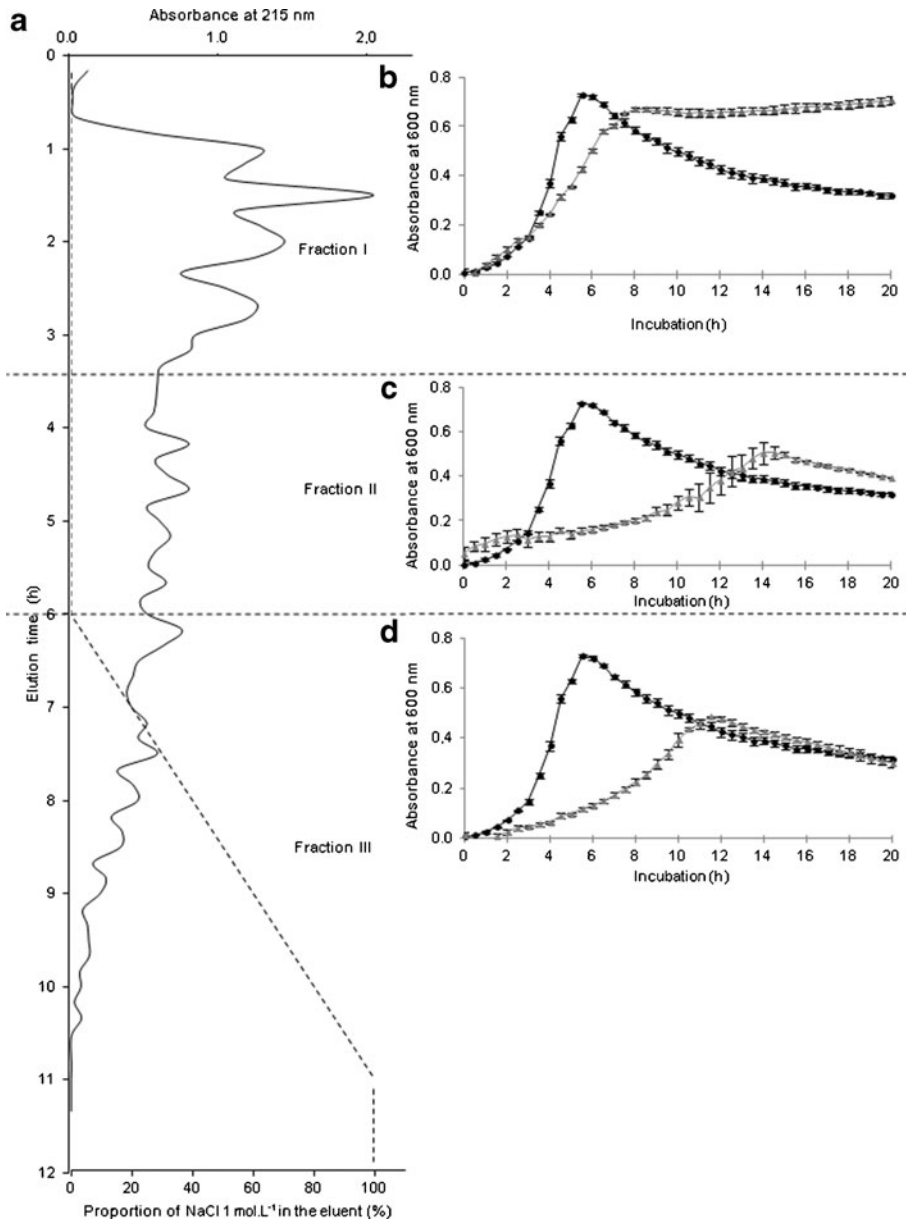
Asiago cheese WSE was further fractionated by cation-exchange liquid chromatography (CEC). A quantity of 0.5 g of cheese WSE lyophilisate was first suspended in 50  $\text{mmol}\cdot\text{L}^{-1}$  citrate buffer and the pH was adjusted to 5.2 using 30% (w/v) NaOH (final volume 30 mL). This preparation was then loaded onto a strong cation-exchanger 15-mL bed volume column (Macro-Prep High S support, Bio-Rad Laboratories, Marnes-la-Coquette, France), equilibrated with 50  $\text{mmol}\cdot\text{L}^{-1}$  citrate buffer, pH 5.2. The flow rate was 15  $\text{mL}\cdot\text{h}^{-1}$ . After 4-h flow-through with 60 mL citrate buffer, the column was eluted with a 5-h linear gradient of 0–1  $\text{mol}\cdot\text{L}^{-1}$  NaCl in citrate buffer. The 215-nm absorbance of eluates was plotted versus elution time and eluates were divided into three fractions according to the plotting depicted in Fig. 1a. The three fractions were dialysed and freeze-dried, as previously described, and their antimicrobial activities were assayed. The AUC of each of the three fractions was calculated.

### 2.4 Bacterial strains and assay of antimicrobial activity

*L. innocua* was considered since (1) it can be used as a biological indicator for *Listeria monocytogenes* because of its similar response to physical, chemical or thermal treatments (Kamat and Nair 1996); (2) it is non-pathogenic in character; and (3) several antimicrobial peptides present in cheeses have been shown to inhibit its growth (Lignitto et al. 2012).

*L. monocytogenes* strain 162 (serotype 1/2a) and *L. innocua* LRGIA 01 both isolated from dairy environment (from cheese brine and from a surface, respectively). *L. monocytogenes* strain 162 was obtained from the Aerial collection (Illkirch, France). Long-term stock cultures were stored at  $-80\text{ }^{\circ}\text{C}$  in a BHI broth supplemented with 15% (v/v) glycerol.

The antibacterial activities of the cheese WSEs against *L. innocua* LRGIA 01 strain and *L. monocytogenes* strain 162 were assayed as follows. To ensure that bacterial growth monitoring was performed in the exponential phase, 9 mL of BHI broth were inoculated with 1 mL of the stock culture and incubated at  $30\text{ }^{\circ}\text{C}$  for 8 h. Then, 1 mL of this first culture was inoculated again with 9 mL of fresh BHI broth and incubated overnight. Finally, a third culture was made from the second one (same conditions) and its turbidity was monitored at 630 nm with a Jenway 6300 spectrophotometer (Bibby



**Fig. 1** **a** Fractionation of Asiago cheese water-soluble extract ripened for 150 days by cation-exchange liquid chromatography (CEC). **b**, **c**, **d** *L. innocua* growth with (dashed triangle) or without (dashed circle) 20 mg lyophilisate.mL<sup>-1</sup> of each peptide fraction obtained following CEC. The results presented are the means±standard deviations (error bars) of three replicates

Scientific Ltd., Staffordshire, UK) until the optical density value corresponding to 10<sup>6</sup> CFU.mL<sup>-1</sup> was reached. For determination of the antibacterial activity, 270 μL of BHI broth alone (control) or supplemented with whole WSEs lyophilisates, 1,000-g.mol<sup>-1</sup> cut-off membranes ultrafiltration permeates of WSEs or freeze-dried CEC fractions of

Asiago cheese WSE (final concentrations: 20 mg of powder per millilitre of culture medium) were mixed either with 30  $\mu\text{L}$  of bacterial inocula or 30  $\mu\text{L}$  of sterile BHI broth (control) in each well of the microplate and incubated at 30 °C for 20 h in a Bioscreen C apparatus (Oy Growth Curves AB Ltd., Helsinki, Finland). The optical density of the culture was monitored at 600 nm ( $\text{OD}_{600}$ ). Negative controls wells (containing 2,400  $\text{IU}\cdot\text{mL}^{-1}$  nisin) were also monitored. Briefly, the  $\text{OD}_{600}$  was determined just prior to incubation ( $t_0$ ) and again after 12-h incubation ( $t_{12}$ ), because  $\text{OD}_{600}$  reached its maximal value after 12 h in control wells. The  $\text{OD}_{600}$  for each replicate at  $t_0$  was subtracted from the  $\text{OD}_{600}$  for each replicate at  $t_{12}$ . The adjusted  $\text{OD}_{600}$  of each control well was then assigned a value of 100% growth. The percent reduction of  $\text{OD}_{600}$  after 12-h incubation was thus determined using the formula:

$$\begin{aligned} & \text{Percent reduction of } \text{OD}_{600} \text{ after 12 h of incubation} \\ & = 100 - \left( \text{OD}_{600} \text{ of test well} / \text{OD}_{600} \text{ of corresponding control well} \right) \times 100. \end{aligned}$$

The percent reduction of maximal growth specific rate ( $\mu$ ) as compared with control wells was also calculated for each cheese extract:  $(\mu \text{ in control well} - \mu \text{ in test well}) / \mu \text{ in control well} \times 100$ . All the antibacterial activity assays were performed in triplicate and data were given as mean  $\pm$  SEM (standard error of the means). The assumption of normal distribution of these data was checked using the Shapiro–Willks test. Differences were tested using a Welch’s one-way ANOVA. The level of significance was set at  $P=0.05$  for all comparisons.

### 3 Results and discussion

#### 3.1 Anti-*Listeria* spp. activity of cheese extracts

The results concerning the inhibition of *Listeria* spp. cells growth in the presence of 20 mg of cheese extracts per mL of BHI broth are presented in Table 1. The three Emmental de Savoie as well as the Asiago cheeses WSEs tested all significantly inhibited *L. innocua* LRGIA01 strain cells growth: the reductions of maximal specific growth rate and of  $\text{OD}_{600}$  after 12-h incubation at 30 °C were both significant. *L. innocua* LRGIA01 inhibition by Asiago cheese WSEs was also reported by Lignitto et al. (2012). However, to our knowledge, antibacterial activity of Emmental de Savoie cheese extracts has never been observed, although the presence in Emmental cheese of 25 mg per 100 g of isracidin ( $\alpha_{s1}$ -CN f(1–23)), an antimicrobial peptide, has been reported by Gagnaire and Léonil (2002). Rizzello et al. (2005) also reported that peptides extracted from Pecorino Romano, Canestrato Pugliese, Crescenza and Caprino del Piemonte cheeses inhibited *L. innocua* DSM 20649 growth.

Interestingly, Emmental de Savoie cheese WSEs also inhibited *L. monocytogenes* strain 162 growth, although to a lesser extent than *L. innocua* LRGIA01. This was also observed with Asiago cheese (Lignitto et al. 2012). In the present study, about 50% inhibition of *L. monocytogenes* strain 162 growth was observed when Emmental de Savoie cheese WSE lyophilisates were added at a 20  $\text{mg}\cdot\text{mL}^{-1}$  concentration in

**Table 1** Influence of cheese extracts lyophilisates addition on the growth of *L. innocua* LRGIA01 and *L. monocytogenes* strain 162 growth at 30 °C in BHI broth

Cheese		Asiago d'Allevo	Emmental de Savoie		
Age (days)		150	97	114	149
<i>L. innocua</i> LRGIA01	Proportion (%) of reduction of OD <sub>600</sub> after 12-h incubation	53±2 <sup>a</sup>	56±1 <sup>a, b</sup>	61±1 <sup>b</sup>	68±1 <sup>c</sup>
	Proportion (%) of reduction of growth rate	57	44	86	70
<i>L. monocytogenes</i> strain 162	Proportion (%) of reduction of OD <sub>600</sub> after 12-h incubation	n.d.	45±6 <sup>a</sup>	39±1 <sup>a</sup>	61±1 <sup>b</sup>
	Proportion (%) of reduction of growth rate		70	88	77

Cheese extracts contain molecules with a molecular mass ranging from ~100–500 to 10,000 g.mol<sup>-1</sup>

Samples with same letters are not significantly different ( $P < 0.05$ )

n.d. not determined. The results presented are the means of three replicates±standard error of the mean

BHI broth. In order to estimate whether this can be of any practical significance, it might be useful to indicate that these compounds are present at ~8 times higher concentration (i.e. ~150 g.L<sup>-1</sup>) in the aqueous phase of these cheeses.

Antimicrobial peptides present in Emmental de Savoie as well as Asiago cheese extracts could be bacteriocins as well as antimicrobial fragments of caseins. In order to get information on the size of peptides inhibiting *L. innocua* growth, the antimicrobial activity of cheese extracts was assayed following their ultrafiltration onto 1,000-g.mol<sup>-1</sup> cut-off membranes to remove peptides with a larger molecular mass (Table 2). The relative contribution of peptides with a molecular mass lower than 1,000 g.mol<sup>-1</sup> to the overall antimicrobial activity of whole cheese extracts was determined by calculating the ratio between the percentage of OD<sub>600</sub> reduction after 12-h incubation at 30 °C in the presence of each cheese extract filtrate (Table 2) and the corresponding percentage in the presence of each whole cheese extract (Table 1). This allowed thus to estimate that peptides with a molecular mass lower than 1,000 g.mol<sup>-1</sup> accounted for 100±2% of the

**Table 2** Influence of cheese extracts permeates lyophilisates addition on the growth of *L. innocua* LRGIA 01 growth at 30 °C in BHI broth

Cheese		Asiago d'Allevo	Emmental de Savoie	
Age (days)		150	97	114
Proportion (%) of reduction of OD <sub>600</sub> after 12-h incubation		58±1 <sup>a</sup>	61±1 <sup>a</sup>	26±2 <sup>b</sup>
Proportion (%) of reduction of growth rate		60	46	91

Cheese extracts permeates contain molecules with a molecular mass ranging from ~100–500 to ~1,000 g.mol<sup>-1</sup>

Samples with same letters are not significantly different ( $P < 0.05$ ). The results presented are the means of three replicates±standard error of the mean



total antimicrobial activity of Emmental de Savoie cheese ripened for 97 days and of Asiago cheese ripened for 150-days extracts, respectively, while this percentage of contribution was only  $42\pm 3\%$  in the case of Emmental de Savoie cheese ripened for 114 days. This latter difference was not observed when comparing maximal growth specific rate reductions induced by addition of cheese extracts filtrates and whole cheese extracts: similar results were observed whatever the cheese sample. It can thus be concluded that *L. innocua* growth inhibition was mainly due to peptides with a molecular mass lower than  $1,000 \text{ g}\cdot\text{mol}^{-1}$ . This indicates that the observed antimicrobial activity cannot only be ascribed to intact bacteriocins, known to present a molecular mass exceeding  $3,000 \text{ g}\cdot\text{mol}^{-1}$  (Drider et al. 2006). The hypothesis of the contribution of fragments of caseins is thus more likely. Identified antimicrobial fragments of caseins reviewed by Silva and Malcata (2005) were reported to have a molecular mass varying from 400 to  $6,000 \text{ g}\cdot\text{mol}^{-1}$ . The largest antimicrobial fragments of caseins, such as isracidin ( $\alpha_{s1}\text{CN f}(1-23)$ , average mass= $2,764.23 \text{ g}\cdot\text{mol}^{-1}$  and theoretical pHi=9.99 as calculated from PeptideMass software on expasy.org) or casocidin-I ( $\alpha_{s2}\text{CN f}(150-188)$ , average mass= $4,869.69 \text{ g}\cdot\text{mol}^{-1}$  and theoretical pHi=10.08) were thus absent from ultrafiltrated cheese extracts, which still strongly inhibited *L. innocua* growth. This is consistent with the study of Lignitto et al. (2012) who observed that the concentration of these two peptides in Asiago cheeses was not correlated with their anti-*Listeria* activity. Therefore, they suggested that anti-*Listeria* activity of Asiago cheese extracts was likely also due to other peptides. The present results strongly suggest that these other peptides likely have a molecular mass lower than  $1,000 \text{ g}\cdot\text{mol}^{-1}$ . The studies regarding the size of antibacterial peptides in cheeses are scarce. Pritchard et al. (2010) compared the antibacterial activity of three Cheddar extracts containing peptides with a molecular mass lower than  $5,000$  or  $10,000 \text{ g}\cdot\text{mol}^{-1}$  or exceeding  $10,000 \text{ g}\cdot\text{mol}^{-1}$ . In contrast to

**Table 3** Influence of addition of CEC fractions of Asiago cheese water-soluble extract lyophilisates on the growth of *L. innocua* LRGIA 01 at  $30^\circ\text{C}$  in BHI broth

	Fraction I	Fraction II	Fraction III
Proportion (%) of mass of Asiago cheese lyophilisate initially loaded on CEC column* (w/w)	26	49	25
Proportion (%) of reduction of maximal OD <sub>600</sub>	$9\pm 1^a$	$31\pm 4^b$	$34\pm 1^b$
Proportion (%) of reduction of growth rate	48	82	72
Proportion (%) of peptides with a molecular mass between 200 and $1,000 \text{ g}\cdot\text{mol}^{-1}$ in cheese extracts*	49	87	74
Proportion (%) of peptides with a molecular mass between 1,000 and $10,000 \text{ g}\cdot\text{mol}^{-1}$ in cheese extracts*	51	13	26

Samples with same letters are not significantly different ( $P < 0.05$ ). The results presented are the means of 3 replicates  $\pm$  standard error of the mean

\*The respective proportions of peptides having a molecular mass comprised between 200 and 1,000, and 1,000 and  $10,000 \text{ g}\cdot\text{mol}^{-1}$  were estimated by analysing the chromatograms obtained following the injection of cheese extracts onto a calibrated gel filtration chromatography Superdex Peptide<sup>TM</sup> column, assuming that the areas of peaks resulting from the elution of molecules absorbing at 215 nm corresponded to peptides and were proportional to their mass concentration

what we observed when fractionating Emmental de Savoie and Asiago cheeses extracts, the highest inhibition of *Escherichia coli* and *Bacillus cereus* growth was exerted by extracts containing high molecular mass peptides (exceeding  $10,000 \text{ g}\cdot\text{mol}^{-1}$ ).

### 3.2 *L. innocua* growth inhibitory activity of CEC fractions of Asiago cheese WSE

Although antimicrobial casein fragments identified to date have a high diversity of structure, most of them are cationic. Therefore, Asiago cheese extract was separated into three fractions onto a column filled with a strong cation exchanger. The fraction I contained molecules which were rapidly eluted (likely non-cationic peptides which did not interact with the chromatographic support). The fraction II corresponded to molecules eluted just thereafter and before the beginning of the linear salt gradient applied to elute cationic peptides more strongly interacting with the chromatographic support. The molecules eluted during salt gradient application were pooled in fraction III. The respective proportions of molecules present in the three fractions as well as their *L. innocua* cells growth inhibitory activities are given in Table 3.

When resuspended in BHI broth at the same concentration ( $20 \text{ mg}\cdot\text{mL}^{-1}$ ), all fractions significantly inhibited *L. innocua* LRGIA01 cells growth. However, fractions II and III reduced both maximal  $\text{OD}_{600}$  as well as maximal specific growth rate far more than fraction I (by 82, 72 and 48%, respectively). This suggests thus that *L. innocua* cells growth is more inhibited by cationic peptides than by non-cationic peptides extracted from Asiago cheese. The cationic nature of numerous antimicrobial peptides is due to the facts that their initial contact with the target bacteria is electrostatic and that most bacterial surfaces are anionic (Powers and Hancock 2003). Moreover, the GFC analysis of molecular mass distribution (Table 3) indicates that the two most active fractions (II and III) mainly contain peptides with a molecular mass between 200 and  $1,000 \text{ g}\cdot\text{mol}^{-1}$ . This is consistent with the previous observation that Asiago cheese extract anti-*L. innocua* activity was mainly due to peptides with a molecular mass lower than  $1,000 \text{ g}\cdot\text{mol}^{-1}$ . However, ultrafiltering onto  $1,000 \text{ g}\cdot\text{mol}^{-1}$  cut-off membrane and testing antimicrobial activity of fraction II permeate would be necessary to assess the validity of this statement.

### 3.3 Characterisation of cheeses and their extracts

Several authors reported that the antibacterial activity of cheese extracts is due to casein fragments. As a consequence, they observed that proteolysis accompanying cheese ripening strongly influences the antibacterial activity of cheese extracts. Therefore, proteolysis of the four cheeses was characterised by measuring the WSN/TN ratio and by assessing the proportion of molecules with a molecular mass between 200 and  $1,000 \text{ g}\cdot\text{mol}^{-1}$  in cheese WSEs by GFC analysis (Table 4). WSN/TN ratio of Asiago cheese ripened for 150 days was equal to 22.8% and WSN/TN ratios of Emmental de Savoie cheeses ripened between 97 and 149 days were quite similar (comprised between 23.0% and 26.6%). WSN/TN of a given cheese increases throughout ripening, however in the present study three different Emmental de Savoie cheeses were sampled out in different cheesemaking plants: the

**Table 4** Cheesemaking parameters and proteolysis of Asiago d'Allevio and Emmental de Savoie cheeses

Cheese	Asiago d'Allevio		Emmental de Savoie	
	Semi-hard cheese	Hard cheese	<i>Streptococcus thermophilus</i>	<i>Lactococci, thermophilic lactobacilli</i> and <i>streptococci</i>
Type				
Starter bacteria				
Cooking temperature (°C)	47	53–54		
Age (days)	150	114	97	149
(WSN/TN)×100	22.8	26.6	26.6	23.0
Proportion (%) of peptides with a molecular mass between 200 and 1,000 g.mol <sup>-1</sup> in cheese extracts*	23	55	55	48
Proportion (%) of peptides with a molecular mass between 1,000 and 10,000 g.mol <sup>-1</sup> in cheese extracts*	77	45	45	52

\*The respective proportions of peptides having a molecular mass between 200 and 1,000, and 1,000 and 10,000 g.mol<sup>-1</sup> were estimated by analysing the chromatograms obtained following the injection of cheese extracts lyophilisates onto a calibrated gel filtration chromatography Superdex™ column, assuming that the areas of peaks resulting from the elution of molecules absorbing at 215 nm corresponded to peptides and was proportional to their mass concentration

WSN water-soluble nitrogen, TN total nitrogen

differences regarding milk, cheesemaking and ripening conditions can thus explain that the cheese with the highest WSN/TN ratio (26.6%) was ripened for 97 days, while that with the lowest WSN/TN ratio (23.0%) was ripened for 149 days. Rizzello et al. (2005) noticed that cheeses ripened for a short or medium time contained antibacterial peptides, while those having longer ripening periods such as Parmigiano-Reggiano cheese did not: they suggested that the antimicrobial fragments of caseins released by proteolytic enzymes would be further degraded into inactive fragments when proteolysis exceeded a certain level. More generally, they considered that cheeses containing the highest amount of bioactive peptides had a WSN/TN ratio that ranged from 12% to 24%. However, since WSN is heterogeneous, further characterisation of the composition of WSN is necessary when studying cheese proteolysis. Therefore, WSEs of each cheese were analysed by GFC. This allowed to observe that while between 41% and 55% of peptides present in Emmental de Savoie cheese WSE had a molecular mass between 200 and 1,000  $\text{g}\cdot\text{mol}^{-1}$ , these peptides accounted for 23% in Asiago cheese WSE, while the four cheese samples analysed had almost the same WSN/TN ratio. This is consistent with the known respective characteristics of semi-hard and hard cheeses. The secondary proteolysis in hard cheeses is known to be far more intense: the large- and medium-size peptides resulting from primary proteolysis of caseins are rapidly degraded into oligopeptides and free amino acids. In semi-hard cheeses, secondary proteolysis is less intense and as a consequence more large- and medium-size peptides can accumulate in the water-soluble fraction of these cheeses. In the present study, it is noteworthy that while peptides with a molecular mass lower than 1,000  $\text{g}\cdot\text{mol}^{-1}$  represented 23% of peptides of Asiago cheese WSE, they accounted for  $100\pm 2\%$  of its inhibitory activity of *L. innocua* growth.

#### 4 Conclusion

Peptides present in Emmental de Savoie cheeses were found to significantly inhibit *L. innocua* and *L. monocytogenes* growth. Moreover, most of the peptides inhibiting *L. innocua* present in Emmental de Savoie cheeses as well as the Asiago cheese tested in this study had a molecular mass lower than 1,000  $\text{g}\cdot\text{mol}^{-1}$ . This indicates that the observed anti-*Listeria* activity cannot be ascribed to intact bacteriocins and is more likely due to low molecular mass antimicrobial fragments of caseins. In order to get further insight in the structural characteristics of the peptides inhibiting *L. innocua* present in Asiago cheese, they were fractionated by CEC. The fraction containing non-cationic peptides had a lower antimicrobial activity than the fractions containing cationic peptides, suggesting thus that cationic peptides significantly contributed to the observed effect. Further fractionation by different chromatographic techniques including CEC will thus be performed to identify the antibacterial peptides present in the two Alpine cheeses.

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